

Safety Assessment of Bangle (*Zingiber purpureum* Rosc.) Rhizome Extract: Acute and Chronic Studies in Rats and Clinical Studies in Human

Eishin Kato,[†] Miwa Kubo,[‡] Yasuko Okamoto,[‡] Yoichi Matsunaga,[‡] Hoko Kyo,[§] Nobutaka Suzuki,[§] Kazuo Uebaba,^{||} and Yoshiyasu Fukuyama^{*,‡,§}

[†]Hosoda SHC Co., Ltd., 3-2-21 Miyuki, Fukui 910-0854, Japan

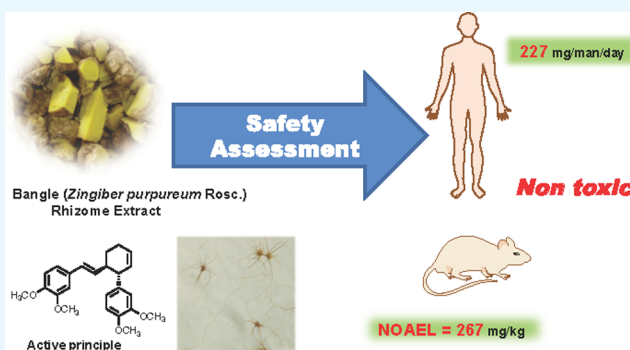
[‡]Faculty of Pharmaceutical Sciences, Tokushima Bunri University, 180 Yamashiro-cho, Tokushima 770-8514, Japan

[§]Department of Complementary and Alternative Medicine Clinical R&D, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan

^{||}Urata Clinic, Medical Corporation HOSPY Group, Uozu, Toyama 937-0805, Japan

Supporting Information

ABSTRACT: Bangle (*Zingiber purpureum* Rosc.) rhizome extract (BRE) contains phenylbutenoid dimers (banglenes), which exert neurotrophic effects and possess the potential capability to regenerate hippocampal neurons in mice. The acute and chronic oral toxicities of BRE powder were evaluated in Sprague–Dawley rats. A dose of BRE powder was estimated to be higher than 2000 mg/kg containing BRE 534 mg/kg as minimum lethal dose in a single-dose oral toxicity study. The no-observed-adverse-effect-level for the BRE powder was 1000 mg/kg/day (BRE 267 mg/kg) in the 90 day oral toxicity study. Four week clinical studies of BRE tablets in humans suggested that the ingestion of BRE tablets within 850 mg/man/day (BRE 227 mg/man/day) was safe for at least 1 month and in a usual manner. The C_{max} , t_{max} , and AUC of *cis*- and *trans*-(*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-enes (*c*- and *t*-banglenes) were calculated after the ingestion of BRE tablets (BRE 227 mg) and were 17.73 and 22.61 ng/mL, 1.8 and 1.8 h, and 71.47 and 95.53 ng/mL/h, respectively.



1. INTRODUCTION

Bangle (Indonesian name), whose species name is *Zingiber purpureum* Rosc., belongs to Zingiberaceae and is a tropical ginger that is widely distributed in Southeast Asia. This ginger is described by the synonyms of *Zingiber cassumunar* Roxb. and *Zingiber montanum* (Koenig) Dietrich in World Spice Plants.¹ Bangle not only has been used as a spice but also has been applied in the traditional Indonesian medicine known as “Jamu” for treatment of fever, headaches, stomach pain, rheumatism, and obesity, and it serves as an ingredient in postpartum herbal medicine.² Previous studies on the rhizomes of *Z. cassumunar*, which is a synonym of *Z. purpureum*, reported the isolation of various types of phenylbutenoids, curcuminoids, and terpenoids.³ In addition, they showed a number of biological activities such as anti-inflammatory and analgesic activities,^{4–8} ovicidal^{9–11} and insecticidal activities,^{12,13} and cytotoxic^{14–17} and antibacterial activities^{18,19} as well as some enzyme inhibition properties.^{20–22} Recently, clinical effects and safety evaluations of the *Z. cassumunar* Roxb. rhizome showed that it was useful for the treatment of pain reduction^{23,24} and estimated an oral no-observed-adverse-

effect level (NOAEL) for *Z. cassumunar* EtOH extract to be 1125 mg/kg body weight/day for rats.²⁵ On the other hand, bangle rhizome contains chemical components similar to those of *Z. cassumunar*, and some compounds were found to have neurotrophin-like activities. Among them, *cis*- and *trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-enes (1) and (2) (named *c*- and *t*-banglenes), dimers of (*E*)-1-(3,4-dimethoxyphenyl)butadiene, were identified as neurotrophic principles in the MeOH extract of the rhizomes of bangle, which exhibited neuritogenic activity in PC12 cells at 25 μ g/mL.²⁶ Banglenes 1 and 2 were found not only to significantly induce neurogenesis of PC12 cells but also to show protective activity against cell death caused by deprivation of serum in primary cultured mouse cortical neurons.²⁶ Additionally, we found that new curcuminoid 3 had an effect on accelerating the prevention of A β 42 aggregation.²⁷ With regard to the in vivo studies, chronic treatment with banglenes 1 and 2 was able to

Received: September 30, 2018

Accepted: November 9, 2018

Published: November 21, 2018

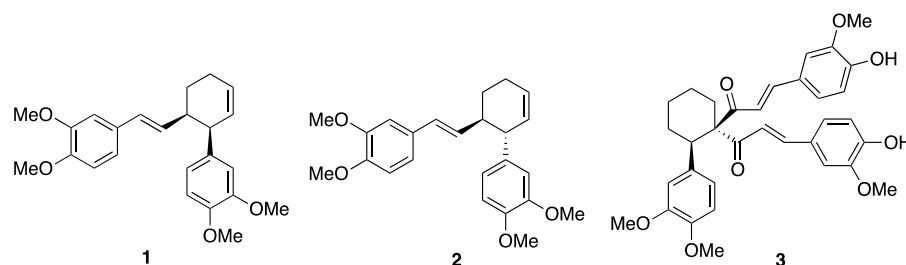


Figure 1. Structures of *c*-banglene (**1**), *t*-banglene (**2**), and curcuminoid (**3**).

Table 1. Body Weights (g, Mean \pm Standard Deviation, SD) of Rats During an Oral Single-Dose Toxicity Study of BRE Powder ($n = 5$)^a

sex	dose (mg/kg)	day 0	day 1	day 2	day 3	day 7	day 10	day 14
male	0	161 \pm 2	181 \pm 4	194 \pm 5	205 \pm 5	245 \pm 6	271 \pm 4	306 \pm 8
	250	159 \pm 4	181 \pm 5	190 \pm 5	201 \pm 6	241 \pm 8	268 \pm 10	304 \pm 14
	700	159 \pm 4	181 \pm 4	192 \pm 4	202 \pm 6	240 \pm 10	266 \pm 13	296 \pm 16
	2000	161 \pm 4	182 \pm 6	191 \pm 7	200 \pm 5	241 \pm 7	269 \pm 8	304 \pm 9
female	0	133 \pm 9	150 \pm 7	157 \pm 8	160 \pm 6	175 \pm 9	188 \pm 12	199 \pm 15
	250	136 \pm 9	150 \pm 10	157 \pm 10	164 \pm 10	177 \pm 13	184 \pm 17	197 \pm 21
	700	131 \pm 8	152 \pm 11	158 \pm 11	163 \pm 9	177 \pm 11	190 \pm 12	202 \pm 13
	2000	136 \pm 8	155 \pm 10	162 \pm 11	167 \pm 11	183 \pm 14	195 \pm 19	208 \pm 19

^aNo significant difference in any treated groups from the control group.

Table 2. Detailed Clinical Observation and Manipulative Test^a for 90 Day Oral Gavage Toxicity Study of BRE Powder (Week 12 of Administration) in Rats ($n = 10$)

parameter	sex dose (mg/kg/day)	male			female		
		0	500	1000	0	500	1000
auditory response		normal	normal	normal	normal	normal	normal
approach response		normal	normal	normal	normal	normal	normal
touch response		normal	normal	normal	normal	normal	normal
tail pinch response		normal	normal	normal	normal	normal	normal
pupillary reflex		pass	pass	pass	pass	pass	pass
aerial righting reflex		pass	pass	pass	pass	pass	pass
landing foot splay (mm) ^a		84 \pm 26	82 \pm 24	79 \pm 17	75 \pm 22	77 \pm 19	65 \pm 20

^aNo significant difference in any treated groups from the control group (mean \pm SD).

enhance hippocampal neurogenesis in dementia model olfactory bulbectomy mice.²⁶ Moreover, chronic treatment of bangle EtOH extract was shown to improve the spatial learning and memory deficits by enhancing hippocampal neurogenesis in senescence-accelerated prone-8 (SAMP8) mice, which are a model for the early stage of Alzheimer's disease.²⁸ Thus, *t*- and *c*-banglenes (**1**, **2**) and curcuminoid **3** have been pharmaceutically verified as active principles that are responsible for the promising improvement of cognitive impairment affected by bangle rhizome EtOH extract (Figure 1).

Although bangle has long been utilized as an edible spice and medicinal supplement in Indonesia and may be developed into a functional food, which has neurotrophic effects on protecting cognitive impairment, there is no documented toxicological study of bangle. Thus, safety assessments are needed to ascertain the degree of safety of bangle preparation. In this paper, we report safety assessment of bangle rhizome extract (abbreviated to BRE) on rats and humans, and pharmacokinetics study of *c*- and *t*-banglenes (**1**, **2**), neurotrophic principles in BRE tablets by measurement of their plasma concentration in humans.

2. RESULTS AND DISCUSSION

2.1. Single-Dose Oral Toxicity Study. No deaths occurred in either sex in any dose group during the 14 day observation period. Therefore, the minimum lethal dose of the BRE powder was estimated to be higher than 2000 mg/kg body weight (BRE 534 mg/kg, *c*- and *t*-banglenes 118 mg/kg) for both males and females. There were no abnormal clinical signs in any animal in either sex in the control group or in any dose group during the observation period. The body weights of males and females in each dose group were comparable to those of the control group during the observation period, and there were no significant differences in either sex from those of the control group during the observation period (Table 1). There were no abnormalities in the external appearance or the cranial, thoracic, or abdominal organs or tissues in any animal in either sex in the control group or in any dose group.

As Matsui et al. report that *c*- and *t*-banglenes were detected in the brains of mice at 0.5 h after oral administration,²⁶ clinical signs such as abnormalities in external appearance, nutritional condition, posture, behavior, and excrement of the rats were checked at 5, 15, 30 min, 1, 2, 4, and 6 h after the administration of 2000 mg/kg body weight of the BRE powder. However, there were no abnormalities.

Table 3. Grip Strength^a and Motor Activity^b for 90 Day Oral Gavage Toxicity Study of BRE Powder (Week 12 of Administration) in Rats ($n = 10$, Mean \pm SD)^c

parameter	sex dose (mg/kg/day)	male			female		
		0	500	1000	0	500	1000
grip strength ^a (g)	forelimb	1976 \pm 128	2040 \pm 127	2126 \pm 190	1367 \pm 249	1349 \pm 196	1300 \pm 221
	hindlimb	1051 \pm 107	1125 \pm 161	1070 \pm 114	756 \pm 112	706 \pm 106	774 \pm 121
motor activity (60 min; count)		1570 \pm 515	1544 \pm 421	1494 \pm 404	1434 \pm 461	1564 \pm 362	1344 \pm 190

^aMeasurement with CPU gauge MODEL-RX-5 (AIKOH Engineering Co., Ltd. Osaka, Japan). ^bMeasurement with motor activity with NS-AS01 (Neuroscience Inc. Tokyo, Japan). ^cNo significant difference in any treated groups from the control group.

Table 4. Body Weights (g)^a for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)

sex	dose (mg/kg/day)	male			female		
		0	500	1000	0	500	1000
day 1		229 \pm 8	229 \pm 7	230 \pm 10	167 \pm 8	167 \pm 8	168 \pm 8
day 7		288 \pm 11	289 \pm 11	290 \pm 15	194 \pm 12	193 \pm 10	196 \pm 11
day 14		353 \pm 17	357 \pm 17	358 \pm 18	214 \pm 15	216 \pm 17	224 \pm 17
day 21		399 \pm 23	409 \pm 25	411 \pm 24	229 \pm 16	229 \pm 20	238 \pm 18
day 28		440 \pm 29	454 \pm 33	454 \pm 28	246 \pm 19	247 \pm 22	252 \pm 20
day 35		476 \pm 35	491 \pm 41	489 \pm 30	263 \pm 18	261 \pm 21	269 \pm 19
day 42		504 \pm 37	518 \pm 45	518 \pm 36	271 \pm 21	269 \pm 28	277 \pm 19
day 49		527 \pm 40	544 \pm 46	545 \pm 39	279 \pm 24	275 \pm 26	282 \pm 22
day 56		549 \pm 41	569 \pm 48	567 \pm 40	287 \pm 22	286 \pm 26	290 \pm 22
day 63		558 \pm 39	589 \pm 54	586 \pm 43	296 \pm 23	295 \pm 28	298 \pm 21
day 70		576 \pm 44	610 \pm 59	603 \pm 44	300 \pm 25	300 \pm 31	308 \pm 23
day 77		591 \pm 46	626 \pm 61	626 \pm 61	306 \pm 27	306 \pm 31	312 \pm 26
day 84		603 \pm 48	639 \pm 66	626 \pm 45	312 \pm 27	310 \pm 30	316 \pm 26
day 91		609 \pm 49	649 \pm 67	634 \pm 47	317 \pm 28	311 \pm 31	318 \pm 26

^aNo significant difference in any treated groups from the control group.

Table 5. Urinalysis^a for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)

sex	dose (mg/kg/day)	male			female		
		0	500	1000	0	500	1000
U.Vol. (mL/24 h)		17.0 \pm 10.5	14.0 \pm 4.1	16.2 \pm 4.1	8.7 \pm 3.7	6.6 \pm 3.7	6.4 \pm 1.7
W.C. (mL/24 h)		38 \pm 12	36 \pm 8	46 \pm 13	32 \pm 6	29 \pm 10	31 \pm 5
Osm.P. (mO/kg) ^b		2008 \pm 608	2104 \pm 415	1895 \pm 453	2081 \pm 602	2290 \pm 585	2080 \pm 248
U-Na (mmol/24 h) ^c		2.2 \pm 0.6	2.2 \pm 0.3	2.0 \pm 0.6	1.3 \pm 0.4	1.0 \pm 0.3	1.0 \pm 0.4
U-K (mmol/24 h) ^c		5.2 \pm 1.6	5.3 \pm 0.6	5.6 \pm 1.3	3.1 \pm 1.2	2.5 \pm 0.8	2.4 \pm 0.6
U-Cl (mmol/24 h) ^{c,d}		3.4 \pm 1.0	3.6 \pm 0.4	3.5 \pm 0.9	2.1 \pm 0.8	1.6 \pm 0.5	1.6 \pm 0.5

^aNo significant difference in any treated groups from the control group. ^bMeasurement with osmotic pressure automatic osmometer OSMO STATION OM-6060 (ARKREY Inc, Kyoto, Japan). ^cMeasurement with ion-selective electrode of Clinical Chemistry Automatic Analyzer TBA-120FR (Canon Medical Systems). ^dU.Vol., urine volume; W.C., water consumption; Osm.P.O, osmotic pressure; U-Na, urine sodium; U-K, urine potassium; U-Cl, urine chloride.

As described above, the nontoxic dose level of BRE powder by single oral administration to rats was 2000 mg/kg, and thus it was suggested that its acute toxicity is extremely weak. This result is similar to that reported by Koontongkaew²⁵ for the *Z. cassumunar* extract.

2.2. Ninety Day Oral Gavage Toxicity Study. A 90 day repeated-dose oral toxicity study of the BRE powder was conducted in the rats. Dose levels were set at 0 (vehicle: water for injection), 500, and 1000 mg/kg/day, and the test formulations were administered orally by gavage once every day using flexible stomach tubes for 91 days.

No deaths occurred, and there were no animals that showed clinical abnormalities caused by administration. In the control group, one male transiently showed fracture of the incisor from week 9 to 10 of administration. In addition, one female showed subcutaneous mass in the perineal area from week 11 of administration.

There were no changes in the detailed clinical observation that were caused by administration of the BRE powder in either sex. Furthermore, the manipulative test, grip strength, and motor activity at week 12 of administration showed no significant differences in either sex from those of the control group during the observation period (Tables 2 and 3).

The body weights of the animals in the dose groups were largely comparable to those of the control group (Table 4). There were no significant differences in any of the treated groups from the control group. The food consumption too of the animals in the dose groups was largely comparable to that of the control group.

There were no ophthalmological changes that were related to the administration of the BRE powder in either sex. The changes that were observed are frequently encountered in rats of this strain and are spontaneous changes. Urinalysis and

Table 6. Hematology^a for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)

sex	male			female		
dose (mg/kg/day)	0	500	1000	0	500	1000
RBC (10^4 /mL) ^a	852 \pm 35	856 \pm 43	851 \pm 28	786 \pm 32	772 \pm 33	773 \pm 32
HGB (g/dL) ^a	15.1 \pm 0.5	15.1 \pm 0.6	15.4 \pm 0.6	14.8 \pm 0.4	14.7 \pm 0.6	14.7 \pm 0.5
HCT (%) ^b	44.5 \pm 1.7	44.5 \pm 1.7	45.1 \pm 1.8	42.3 \pm 1.5	41.8 \pm 1.5	42.1 \pm 1.6
MCV (fL) ^b	52.3 \pm 1.2	51.9 \pm 2.0	53.0 \pm 1.4	53.8 \pm 1.3	54.2 \pm 1.4	54.5 \pm 1.4
MCH (pg) ^b	17.8 \pm 0.4	17.7 \pm 0.8	18.1 \pm 0.5	18.8 \pm 0.4	19.0 \pm 0.6	19.0 \pm 0.5
MCHC (g/dL) ^b	34.0 \pm 0.3	34.1 \pm 0.4	34.2 \pm 0.4	35.0 \pm 0.5	35.0 \pm 0.6	34.9 \pm 0.7
Retic (10^3 /L) ^b	180.5 \pm 46.6	179.2 \pm 20.1	164.7 \pm 34.1	169.5 \pm 34.2	144.7 \pm 31.9	168.4 \pm 46.6
PLT (10^4 /mL) ^b	100.8 \pm 10.3	94.5 \pm 9.3	96.5 \pm 13.1	103.6 \pm 8.1	96.5 \pm 9.7	104.0 \pm 8.0
WBC (10^2 /mL) ^b	89.8 \pm 11.0	85.1 \pm 18.1	94.0 \pm 18.5	59.1 \pm 29.1	52.1 \pm 17.3	48.0 \pm 11.3
LYMP (10^2 /mL) ^b	64.3 \pm 8.2	61.4 \pm 15.6	68.0 \pm 12.9	46.0 \pm 26.4	41.9 \pm 16.4	37.1 \pm 9.0
NEUT (10^2 /mL) ^b	19.8 \pm 6.4	17.6 \pm 5.1	19.6 \pm 8.6	10.0 \pm 3.3	7.3 \pm 3.1	8.3 \pm 2.4
EOS (10^2 /mL) ^b	1.3 \pm 0.4	1.3 \pm 0.5	1.4 \pm 0.4	0.9 \pm 0.4	0.9 \pm 0.4	0.6 \pm 0.2
BASO (10^2 /mL) ^b	0.2 \pm 0.1	0.2 \pm 0.1	0.3 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.0
MONO (10^2 /mL) ^b	3.3 \pm 0.8	3.6 \pm 1.2	4.0 \pm 1.0	1.5 \pm 0.3	1.6 \pm 0.8	1.5 \pm 0.7
LUC (10^2 /mL) ^b	1.0 \pm 0.5	1.0 \pm 0.7	0.8 \pm 0.2	0.5 \pm 0.4	0.4 \pm 0.2	0.4 \pm 0.2
PT (s) ^c	12.5 \pm 0.7	13.0 \pm 1.3	13.4 \pm 1.4	12.0 \pm 0.7	11.8 \pm 0.7	11.6 \pm 0.5
APTT (s) ^c	15.9 \pm 1.2	16.7 \pm 1.7	17.5 \pm 1.5 ^{d,e}	14.6 \pm 1.4	14.3 \pm 1.4	14.2 \pm 0.8
FIB (mg/dL) ^c	309 \pm 59	309 \pm 59	283 \pm 17	187 \pm 19	176 \pm 19	184 \pm 17

^aNo significant difference in any treated groups from the control group. ^bMeasurement with Hematology System, Advia 120 (Siemens Healthcare Diagnostics Inc, Germany). ^cMeasurement with Coagulometer ACL Elite Pro (Instrumentation Laboratory Japan Co. Ltd, Tokyo). ^dSignificantly different from the control by Dunnett test two-side (*: less than 0.05). ^eLYMP, lymphocytes; NEUT, neutrophils; EOS, eosinophils; BASO, basophis; MONO, monocytes; LUC, large unstained cells.

Table 7. Blood Chemistry^a for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)^b

sex	male			female		
dose (mg/kg/day)	0	500	1000	0	500	1000
AST (IU/L)	66 \pm 18	60 \pm 8	59 \pm 6	58 \pm 4	60 \pm 17	54 \pm 4
ALT (IU/L)	28 \pm 4	29 \pm 3	28 \pm 3	25 \pm 3	31 \pm 16	25 \pm 4
LDH (IU/L)	40 \pm 10	36 \pm 5	34 \pm 6	32 \pm 5	40 \pm 25	34 \pm 6
ALP (IU/L)	306 \pm 30	303 \pm 62	315 \pm 75	154 \pm 36	136 \pm 36	127 \pm 29
T-CHO (mg/dL)	85 \pm 20	86 \pm 16	86 \pm 22	70 \pm 10	83 \pm 21	90 \pm 14 ^d
TG (mg/dL)	71 \pm 37	75 \pm 23	84 \pm 25	23 \pm 10	31 \pm 18	27 \pm 12
PL (mg/dL)	126 \pm 23	129 \pm 17	132 \pm 22	145 \pm 22	158 \pm 32	167 \pm 16
T-BIL (mg/dL)	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0
GLU (mg/dL)	123 \pm 8	134 \pm 18	142 \pm 21 ^d	129 \pm 9	129 \pm 16	129 \pm 12
BUN (mg/dL)	11 \pm 2	12 \pm 1	12 \pm 1	14 \pm 2	14 \pm 2	14 \pm 2
CRNN (mg/dL)	0.29 \pm 0.03	0.29 \pm 0.03	0.27 \pm 0.03	0.33 \pm 0.02	0.32 \pm 0.03	0.31 \pm 0.03
Na (mM)	146 \pm 1	145 \pm 1	145 \pm 1	144 \pm 1	144 \pm 1	144 \pm 1
K (mM)	3.7 \pm 0.2	3.8 \pm 0.2	3.9 \pm 0.1	3.5 \pm 0.1	3.5 \pm 0.2	3.6 \pm 0.2
Cl (mM)	107 \pm 1	107 \pm 1	107 \pm 1	108 \pm 2	108 \pm 2	108 \pm 1
Ca (mg/dL)	10.2 \pm 0.4	10.5 \pm 0.4	10.5 \pm 0.3	10.1 \pm 0.2	10.5 \pm 0.5 ^d	10.6 \pm 0.3 ^d
P (mg/dL)	5.5 \pm 0.6	5.6 \pm 0.6	6.0 \pm 0.5	5.1 \pm 0.7	4.8 \pm 0.8	5.1 \pm 0.9
TP (g/dL)	6.3 \pm 0.3	6.4 \pm 0.3	6.4 \pm 0.4	6.7 \pm 0.4	7.0 \pm 0.5	6.9 \pm 0.3
ALB (g/dL)	3.1 \pm 0.1	3.2 \pm 0.1	3.2 \pm 0.1	3.8 \pm 0.3	4.1 \pm 0.4	4.0 \pm 0.1
A/G ^c	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	1.3 \pm 0.1	1.4 \pm 0.1	1.3 \pm 0.1

^aMeasurement with Clinical Chemistry Automatic Analyzer TBA-120FR (Toshiba Medical Systems Corporation, Tokyo, Japan). ^bNo significant difference in any treated groups from the control group. ^cA/G ratio (A/G) was calculated from the total protein and albumin. ^dSignificantly different from the control by Dunnett test two-side (*: less than 0.05).

water intake showed no test-article-related changes in either sex (Table 5).

There were no hematology-related changes in either sex (Table 6). A significant prolongation of activated partial thromboplastin time (APTT) in the male 1000 mg/kg group was recorded, but this change was within the range of physiological fluctuation since it was within the range of the historical background data in the test facility.

Blood chemistry data are indicated in Table 7. There were no test-article-related changes in either sex. Significantly high values in comparison with the values in the control group were recorded for glucose in the males in the 1000 mg/kg group, total cholesterol in the females in the 1000 mg/kg group, and calcium in the females in the 500 and 1000 mg/kg groups. However, these values were thought to be within the range of physiological variations since they are largely within the range of the historical background data of the test facility.

Table 8. Absolute Organ Weight for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)^a

sex	male			female		
dose (mg/kg/day)	0	500	1000	0	500	1000
FBW* (g)	582 \pm 48	617 \pm 68	602 \pm 47	297 \pm 27	294 \pm 29	300 \pm 26
brain (g)	2.27 \pm 0.08	2.25 \pm 0.10	2.31 \pm 0.10	2.04 \pm 0.11	1.96 \pm 0.07	1.97 \pm 0.08
pituitary (g)	13.2 \pm 1.3	14.7 \pm 1.2	14.5 \pm 1.9	17.3 \pm 2.7	16.1 \pm 2.9	17.3 \pm 2.0
thyroid-RL (mg)	25.0 \pm 5.3	25.8 \pm 4.8	25.2 \pm 4.4	19.5 \pm 4.2	20.1 \pm 4.1	19.4 \pm 2.4
Sa.G.-RL (mg)	744 \pm 101	800 \pm 58	802 \pm 97	470 \pm 72	447 \pm 45	454 \pm 48
thymus (mg)	268 \pm 61	301 \pm 62	264 \pm 47	261 \pm 50	240 \pm 57	274 \pm 68
heart (g)	1.62 \pm 0.13	1.76 \pm 0.18	1.80 \pm 0.22	0.95 \pm 0.09	0.95 \pm 0.07	1.00 \pm 0.10
lung (g)	1.63 \pm 0.07	1.67 \pm 0.15	1.72 \pm 0.16	1.13 \pm 0.10	1.17 \pm 0.11	1.14 \pm 0.06
liver (g)	14.58 \pm 1.92	16.64 \pm 2.27	16.84 \pm 2.12 ^b	7.66 \pm 1.18	7.64 \pm 0.92	8.25 \pm 0.77
spleen (g)	0.85 \pm 0.09	0.86 \pm 0.15	0.81 \pm 0.14	0.51 \pm 0.06	0.48 \pm 0.07	0.50 \pm 0.07
kidney-RL (g)	3.46 \pm 0.23	3.77 \pm 0.36 ^b	3.81 \pm 0.20 ^{b,c}	1.98 \pm 0.18	1.95 \pm 0.18	2.03 \pm 0.17
adrenal-RL (mg)	59 \pm 11	64 \pm 9	60 \pm 6	69 \pm 17	72 \pm 6	71 \pm 13
testis-RL (g)	3.57 \pm 0.13	3.77 \pm 0.31	3.80 \pm 0.27			
prostate (g)	1.36 \pm 0.18	1.31 \pm 0.11	1.31 \pm 0.1			
sem. ves. (g)	1.61 \pm 0.19	1.64 \pm 0.17	1.65 \pm 0.16			
ovary-RL (mg)				85.2 \pm 14.0	78.9 \pm 16.6	78.9 \pm 16.6
uterus (mg)				698 \pm 115	610 \pm 80	610 \pm 80

^aNo significant difference in any treated groups from the control group. ^bSignificantly different from the control by Dunnett test two-side (*: less than 0.05). ^cFBW, final body weight after fast; Sa.G.-RL, right and left of the salivary gland.

Table 9. Relative Organ Weight for 90 Day Oral Gavage Toxicity Study of BRE Powder ($n = 10$, Mean \pm SD)^{a,b,c}

sex	male			female		
dose (mg/kg/day)	0	500	1000	0	500	1000
FBW* (g)	582 \pm 48	617 \pm 68	602 \pm 47	297 \pm 27	294 \pm 29	300 \pm 26
brain (g)	0.39 \pm 0.03	0.37 \pm 0.03	0.39 \pm 0.03	0.69 \pm 0.06	0.67 \pm 0.06	0.69 \pm 0.66
pituitary (g)	2.3 \pm 0.3	2.4 \pm 0.2	2.4 \pm 0.2	5.8 \pm 0.5	5.5 \pm 1.4	5.8 \pm 0.5
thyroid-RL (mg)	4.3 \pm 0.8	4.2 \pm 0.7	4.2 \pm 0.7	6.6 \pm 1.3	6.8 \pm 1.1	6.5 \pm 0.7
Sa.G.-RL (mg)	128 \pm 13	131 \pm 11	133 \pm 13	158 \pm 13	153 \pm 14	152 \pm 14
thymus (mg)	46 \pm 10	49 \pm 11	44 \pm 7	88 \pm 18	82 \pm 20	91 \pm 20
heart (g)	0.28 \pm 0.01	0.29 \pm 0.03	0.30 \pm 0.02	0.32 \pm 0.02	0.33 \pm 0.03	0.33 \pm 0.03
lung (g)	0.28 \pm 0.02	0.29 \pm 0.03	0.30 \pm 0.02	0.38 \pm 0.01	0.40 \pm 0.03	0.38 \pm 0.02
liver (g)	2.50 \pm 0.16	2.69 \pm 0.14*	2.80 \pm 0.24**	2.57 \pm 0.20	2.60 \pm 0.19	2.75 \pm 0.09*
spleen (g)	0.15 \pm 0.01	0.14 \pm 0.02	0.14 \pm 0.02	0.17 \pm 0.02	0.16 \pm 0.02	0.17 \pm 0.02
kidney-RL (g)	0.60 \pm 0.03	0.61 \pm 0.06	0.64 \pm 0.04	0.67 \pm 0.04	0.67 \pm 0.06	0.68 \pm 0.04
adrenal-RL (mg)	10 \pm 2	10 \pm 2	10 \pm 1	23 \pm 5	25 \pm 3	24 \pm 4
testis-RL (g)	0.62 \pm 0.05	0.62 \pm 0.06	0.64 \pm 0.05			
prostate (g)	0.23 \pm 0.03	0.21 \pm 0.02	0.22 \pm 0.03			
sem. ves. (g)	0.28 \pm 0.04	0.27 \pm 0.04	0.28 \pm 0.03			
ovary-RL (mg)				28.9 \pm 5.5	27.4 \pm 5.1	26.1 \pm 4.1
uterus (mg)				237 \pm 44	233 \pm 52	205 \pm 32

^aNo significant difference in any treated groups from the control group. ^b*, ** significantly different from the control by Dunnett test two-side (*: less than 0.05; **: less than 0.01). ^cFBW, final body weight after fast; Sa.G.-RL, right and left of the salivary gland.

The results of the organ weight measurements are shown in Tables 8 and 9 (relative organ weights). For the liver, significantly high values or a tendency toward a high value in the absolute weight and a significantly high value in the relative weight were recorded in the males in the 500 and 1000 mg/kg groups, respectively, and in the females in the 1000 mg/kg group. For the kidneys, a significantly high value in the absolute weight and a tendency toward high value in the relative weight were recorded in the males in the 1000 mg/kg group. However, these changes were thought to be within the range of the physiological variations since there were no changes related to the administration of the test article in any parameter of the blood chemistry examination, there were no changes in the liver or the kidneys that were related to the administration of the test article in the histopathological

examination, and the changes were largely within the range of the historical background data of the test facility for untreated animals. Otherwise, the absolute kidney weight in the males in the 500 mg/kg group was significantly high, but it was not thought to be a significant change since the relative weight was comparable to that of the control group.

There were no necropsy-related changes in any organ or tissue in either sex, although incidental changes were noticed based on their incidence. There were no histopathology-related changes in any organ or tissue in either sex, although incidental changes in the animals of this strain were observed at each time point.

Accordingly, the no-observed-adverse-effect-level (NOAEL) of BRE powder was judged to be 1000 mg/kg/day (BRE 267 mg/kg/day, *c*- and *t*-banglenes 59 mg/kg/day) for both males

Table 10. Ingestion of BRE Tablet in Adult Subject for 4 Weeks

parameter	BRE	170 mg (<i>n</i> = 8, mean \pm SD)		850 mg (<i>n</i> = 7, mean \pm SD)	
	week	0	4	0	4
SBP	(mmHg)	121 \pm 18	109 \pm 42	138 \pm 16	130 \pm 13
DBP	(mmHg)	76 \pm 9	77 \pm 11	90 \pm 12	85 \pm 10
pulse	(bpm)	83 \pm 12	89 \pm 8	77 \pm 16	74 \pm 7
Blood Count and Hemogram					
WBC	(10 ³ /mL)	55 \pm 11	9 \pm 13	69 \pm 40	55 \pm 15
RBC	(10 ⁴ /mL)	465 \pm 32	447 \pm 39	471 \pm 44	493 \pm 37
Hb	(g/dL)	14.1 \pm 1.2	13.4 \pm 1.4	14.8 \pm 1.1	15.4 \pm 0.9
Hb	(%)	88 \pm 8	84 \pm 9	92 \pm 7	96 \pm 5
HCT	(%)	41.4 \pm 2.6	40.4 \pm 2.9	44.8 \pm 3.2	46.8 \pm 2.9
MCV	(fL)	89.0 \pm 2.4	90.4 \pm 2.7	95.3 \pm 4.1	95.2 \pm 3.3
MCH	(pg)	30.3 \pm 1.1	30.0 \pm 1.3	31.4 \pm 1.2	31.2 \pm 1.0
MCHC	(%)	34.1 \pm 1.0	33.2 \pm 1.1	33.0 \pm 0.4	32.8 \pm 0.4
PLT	(10 ⁴ /mL)	29.9 \pm 5.4	28.5 \pm 4.6	24.9 \pm 6.4	25.9 \pm 6.9
Neut	(%)	56.1 \pm 9.3	58.1 \pm 8.8	64.5 \pm 11.4	57.2 \pm 10.9
Eo	(%)	2.1 \pm 1.8	2.1 \pm 1.4	3.5 \pm 2.3	3.9 \pm 3.3
Ba	(%)	0.5 \pm 0.3	0.4 \pm 0.2	0.7 \pm 0.3	0.8 \pm 0.2
Ly	(%)	36.5 \pm 8.3	33.3 \pm 8.4	25.2 \pm 9.8	32.5 \pm 7.1
Mo	(%)	4.8 \pm 1.3	6.0 \pm 2.2	6.1 \pm 1.1	5.6 \pm 1.4
Biochemical Test					
total protein	(g/dL)	7.7 \pm 0.3	7.4 \pm 0.4	7.4 \pm 0.3	7.6 \pm 0.3
albumin	(g/dL)	4.9 \pm 0.2	4.7 \pm 0.1	4.5 \pm 0.2	4.8 \pm 0.2
AST	(IU/L)	18 \pm 3	22 \pm 9	19 \pm 4	22 \pm 6
ALT	(IU/L)	14 \pm 4	20 \pm 12	16 \pm 4	20 \pm 9
ALP	(IU/L)	208 \pm 30	195 \pm 27	184 \pm 47	201 \pm 49
γ -GTP	(IU/L)	17 \pm 6	18 \pm 10	28 \pm 11	31 \pm 13
ChE	(IU/L)	330 \pm 81	311 \pm 90	323 \pm 54	363 \pm 44
T-Bil	(mg/dL)	0.6 \pm 0.2	0.6 \pm 0.3	0.6 \pm 0.1	0.6 \pm 0.3
T-Chl	(mg/dL)	193 \pm 41	183 \pm 29	195 \pm 39	201 \pm 29
TG	(mg/dL)	126 \pm 68	106 \pm 51	131 \pm 77	135 \pm 4
HDL	(mg/dL)	76 \pm 17	64 \pm 14	65 \pm 18	66 \pm 16
LDL	(mg/dL)	105 \pm 34	100 \pm 32	113 \pm 25	120 \pm 16
BUN	(mg/dL)	11.1 \pm 2.9	10.3 \pm 3.4	12.6 \pm 2.4	12.3 \pm 2.7
Cr	(mg/dL)	0.72 \pm 0.19	0.72 \pm 0.17	0.90 \pm 0.24	0.85 \pm 0.22
Na	(mEq/L)	142 \pm 1	142 \pm 2	144 \pm 2	143 \pm 2
K	(mEq/L)	4.4 \pm 0.3	4.1 \pm 0.5	4.5 \pm 0.6	4.3 \pm 0.4
Cl	(mEq/L)	103 \pm 1	105 \pm 2	104 \pm 1	103 \pm 1
PT	(s)	11.6 \pm 0.4	11.4 \pm 0.5	11.5 \pm 0.2	11.1 \pm 0.3
PT	(%)	94 \pm 7	99 \pm 10	97 \pm 5	104 \pm 6
PT	(INR)	1.03 \pm 0.03	1.01 \pm 0.04	1.02 \pm 0.02	0.99 \pm 0.03
APTT	(s)	28.3 \pm 3.1	30.0 \pm 2.4	27.6 \pm 2.0	27.1 \pm 2.1
Urinalysis					
urobilinogen					
urine protein				^{a,b}	
urine sugar					
uric blood					
urine pH		5.8 \pm 0.5	5.8 \pm 0.7	5.6 \pm 0.5	5.7 \pm 0.8

^aOne subject was 1+. ^bSBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; Neut, neutrophil; Eo, eosinophil granulocyte; Ba, basophil; Ly, lymphocyte; Mo, monocyte; ChE, cholinesterase; T-Bil, total bilirubin; T-Chl, total cholesterol; TG, triglyceride; HDL, HDL cholesterol; LDL, LDL cholesterol; BUN, blood urea nitrogen; Cr, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time.

and females in this study. BRE gave the same result as Koontongkaew et al.²⁵ reported for *Z. cassumunar* extract, although the dose was lower.

2.3. Clinical Study of Bangle Extract for 4 Weeks.

There were no significant differences in the blood pressure and pulse rate of the quintuple group compared to the usual group (Table 10). Variations within the normal limits were noticed

for mean corpuscular volume, and mean corpuscular hemoglobin concentration in the blood count, and hemogram (Table 10). No significant differences between the groups in white blood cells, red blood cells, platelet, hemoglobin, or hematocrit were noted.

There were no significant differences in the blood biochemical tests between the quintuple group and the usual

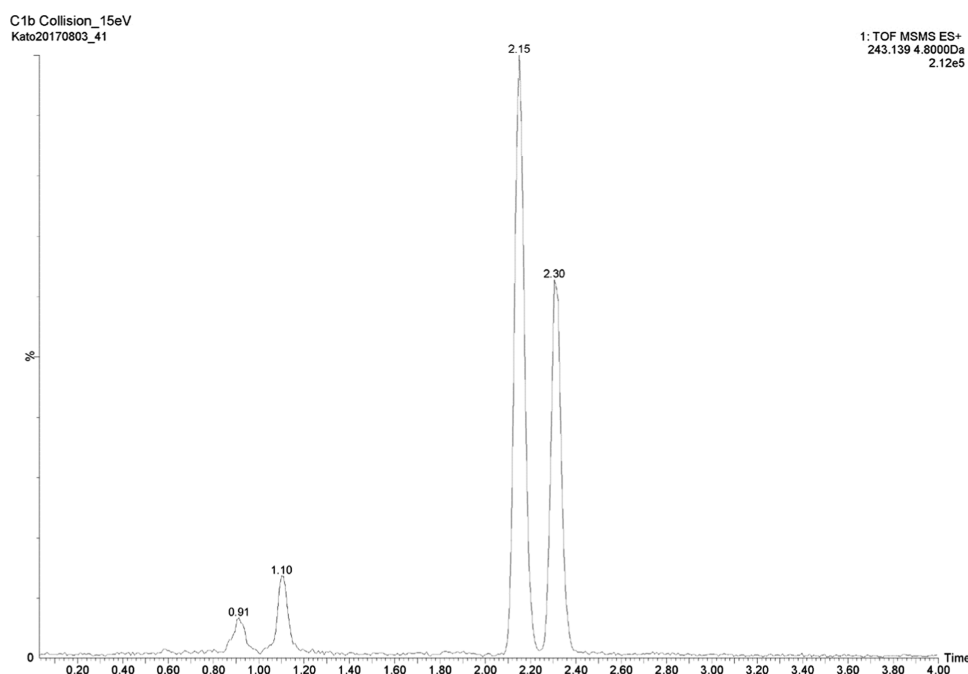


Figure 2. UPLC-MS/MS chromatogram of the plasma at 1 h after the ingestion of BRE tablets (BRE 227 mg). The retention times at 2.15 and 2.30 min are assigned to *t*- and *c*-banglenes (1 and 2), respectively.

group (Table 10). Differences were noted in PT and APTT, but the variations were within the normal limits.

The blood triglyceride values of two subjects in the quintuple group before ingestion were over reference. However, it was determined that ingestion did not influence this value since their blood triglyceride was still over the reference value after 4 weeks. The urinalysis did not show abnormal results although the urine protein of one subject in the quintuple group was 1+ before ingestion.

Consequently, it was concluded that the ingestion of up to 850 mg/day BRE powder (BRE dose of 227 mg/day) is safe in a usual manner for at least 1 month.

2.4. Concentrations of *c*- and *t*-Banglenes in Plasma.

The plasma of subjects was analyzed with ultra performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) in order to confirm the transfer of banglene into the body after the ingestion of 10 BRE tablets (BRE 227 mg, 50 mg of *t*- and *c*-banglenes). Peaks at 3.6 and 3.9 min on the UPLC-MS/MS chromatogram (Figure 2) of the plasma at 1 h after ingestion were due to *t*- and *c*-banglenes (1 and 2), of which the concentrations were 13.93 ± 8.13 and 12.51 ± 7.03 ng/mL (mean \pm SD), respectively. The plasma concentration–time profiles of *t*- and *c*-banglenes after the ingestion showed a maximum at 1.5 h (20.87 ± 8.62 and 16.46 ± 6.67 ng/mL) and decreased at 2 h (16.98 ± 11.01 and 12.92 ± 7.99 ng/mL) and 6 h (2.89 ± 1.00 and 1.91 ± 0.54 ng/mL), finally reaching 0.52 ± 0.30 and 0.42 ± 0.24 ng/mL at 24 h, respectively (Figure 3). The maximum concentrations (C_{\max}), the times at maximum concentration (t_{\max}), the areas under the plasma concentration–time curve (AUC), and the mean residence times (MRTs) calculated by no compartment analysis are indicated in Table 11.

Both banglenes were promptly absorbed after ingestion of the BRE powder, and their t_{\max} value was 1.8 ± 0.3 h (mean \pm SD). Further, the MRTs of *c*- and *t*-banglenes were 4.36 ± 1.17 and 4.45 ± 1.24 h, so it is clear that the pharmacokinetics of both banglenes are not different, nor do they show retentively.

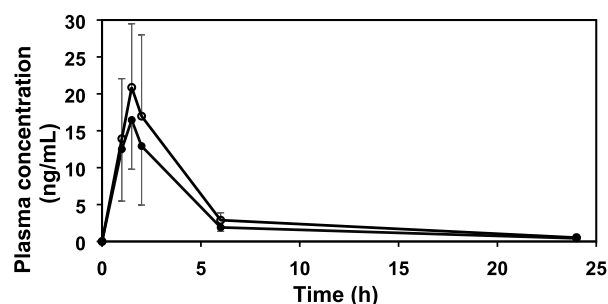


Figure 3. Plasma concentration–time curves of *c*-banglene (closed circle) and *t*-banglene (open circle) in five human healthy subjects (32–54 years; four males and a female) after ingesting 10 BRE tablets. The concentrations are represented as mean, and the bars illustrate SD.

Table 11. Pharmacokinetic Parameters of *c*- and *t*-Banglenes in Subject Plasma after Ingestion of 10 BRE Tablets ($n = 5$, Mean \pm SD)^a

unaltered substance	<i>c</i> -banglene	<i>t</i> -banglene
C_{\max} (ng/mL)	17.73 ± 5.61	22.61 ± 6.70
t_{\max} (h)	1.8 ± 0.3	1.8 ± 0.3
AUC_{1-24h} (ng·h/mL)	71.47 ± 26.17	95.53 ± 36.89
$AUMC_{1-24h}$ (ng·h ² /mL)	297.26 ± 88.69	405.74 ± 127.32
MRT (h)	4.36 ± 1.17	4.45 ± 1.24

^aSubject: four males (32–54 years) and a female (50 years).

This result is analogous to the pharmacokinetic evaluation in the plasma of mice that was reported by Matsui et al. (Matsui, 2012).²⁶ Accordingly, it has been suggested that both banglenes can move from the blood to the brain. The C_{\max} of *c*- and *t*-banglenes reached 17.73 ± 5.61 and 22.61 ± 6.70 ng/mL and their AUCs were 71.47 ± 26.17 and 95.53 ± 36.89 ng·h/mL (mean \pm SD), respectively. These differences in the values for *c*- and *t*-banglenes (C_{\max} : $t/c = 1.27$; AUC: $t/c =$

1.34) are determined by the ratio of *c*- and *t*-ones (1.24) in the tablet.

3. CONCLUSIONS

In this study, a safety assessment of the acute and chronic oral toxicities of BRE for SD rats was conducted, and clinical trials in humans were evaluated. In the 90 day oral toxicity test, no deaths occurred, and there were no animals that showed clinical abnormalities, which were thought to be caused by administration. In addition, a 4 week clinical study of BRE tablets in humans suggested that the ingestion of BRE tablets within BRE powder 850 mg/person/day (BRE 227 mg/person/day) is safe in the usual way in at least 1 month. These studies suggest that BRE tablets are tolerable for single- and long-term administrations. According to our assessment, BRE contains *c*- and *t*-bangles, which exert neurotrophic effects, enhance hippocampal neurogenesis, and improve spatial learning and memory deficits and therefore has the potential to be considered as a useful food for cognitive deficits as long as its intake does not exceed 227 mg/person/day.

4. MATERIALS AND METHODS

4.1. General. UPLC-MS/MS analysis was carried out by using SYNAPT G2-Si HDMS system attached ACQUITY UPLC I-Class and QuanLynx (Waters, Tokyo). Evaporation was achieved with Conbinievapo C10 Light (Biochromato). Centrifuge was done by MCF-2360 (LMS, Tokyo, Japan). Ultrapure water was prepared by Millipore Direct-Q 3UV (Merck Millipore).

4.2. Reagents. Water for injection was obtained from Otsuka Pharmaceutical Factory, Inc. (Naruto, Japan). Methanol was purchased from Kanto Chemical (Tokyo, Japan). 2-Propanol was purchased from Nacalai Tesque (Kyoto, Japan). Acetonitrile was purchased from Kishida Chemical (Osaka, Japan). Ammonium acetate was obtained from Sigma-Aldrich Japan (Tokyo). These reagents were of analytical grade. Acetonitrile for UPLC-MS was purchased from Life Technologies Japan (Yokohama).

4.3. Materials. Cosmospin Filter G (0.2 μm) was purchased from Nacalai Tesque (Kyoto, Japan). The Acquity UPLC BEH C18, 2.1 \times 100 mm, 1.7 μm separation column and Oasis PRiMe HLB Extraction Cartridge were purchased from Nihon Waters K. K. (Tokyo, Japan).

4.4. Powder and Tablets of Bangle Rhizome Extract as Test Substance. The sliced bangle rhizomes, which were cultivated at Ponrogo in Indonesia, were heated and immersed in ethanol for 3 days. The mixture was filtered and concentrated in NHI (Bandon, Indonesia). To the concentrate imported from Indonesia, ethanol, sucrose fatty acid ester (Mitsubishi-chemical Foods Corporation, Tokyo, Japan), high branched cyclodextrin (Glico Nutrition Co., Ltd. Osaka, Japan), and water were added. The mixture was distilled off ethanol under reduced pressure and lyophilized to produce BRE powder. This powder contained 26.7% BRE and 5.9% *c*- and *t*-bangles (*t/c* = 1.24) on the chromatogram by HPLC analysis.

BRE tablets (200 mg/tablet) containing 85 mg/tablet of BRE powder (22.7 mg/tablet of BRE containing 5 mg/tablet of *c*- and *t*-bangles), sucrose fatty acid ester, and dextrin were made from powder, pregelatinized starch (Asahi Kasei Chemicals, Tokyo, Japan), Xantan gum (Ina Food Industry,

Nagano, Japan), silicon dioxide (CDSL, Japan, Tokyo), and shellac (Gifu Shellac Manufacturing, Gifu, Japan).

4.5. Preparation of *c*- and *t*-Bangles (1) and (2). 1-(3,4-Dimethoxyphenyl)buta-1,3-diene was heated in the presence of hydroquinone in toluene and separated by silica gel column chromatography to yield *c*- and *t*-bangles according to the modified method of Tuntiwachwuttikul et al.²⁹

4.6. Test Animals. For single-dose and 90 day oral gavage toxicity studies, Sprague–Dawley strain SPF rats (CrI:CD-(SD)) (Atsugi Breeding Center, Charles River Laboratories Japan, Inc.) were purchased and quarantined/acclimated to the test environment.

4.7. Husbandry Conditions. The single-dose and 90 day oral toxicity studies were conducted at BoZo Research Center Inc. (Tokyo, Japan) in compliance with Good Laboratory Standard Regulations (Ordinance No. 21 of the Ministry of Health and Welfare, Japan)^{30–32} in accordance with OECD test guidelines³³ and with acts/guidelines relating to animal welfare in Japan. In addition, the studies were conducted under the approval of the Institutional Animal Care and Use Committee of the test facility (Approved No. G150068), and the test facility has been authorized by the AAALAC International.

The animals were housed in an animal room and individually in the area, and this housing was created by inserting a divider plate into the plastic cages (Hanyu Seimitsu Co. Ltd., Saitama, Japan). For each area, bedding (ALPHA-dri, Shepherd Specialty Papers, Inc.) was provided, and enrichment (Harlan Laboratories Japan Co. Ltd.) was given once a week during the period of animal use. Animals were allowed free access to pelleted diet CR-LPF (irradiation-sterilized, Oriental Yeast Co., Ltd. Tokyo Japan) using stainless steel feeders and tap water (Gotemba City Water, via an automatic water-supply system).

4.8. Single-Dose Oral Toxicity Study. The test animals (6 weeks old; *n* = 5/sex/group) were administered a single oral dose of BRE powder at 0, 250, 700, and 2000 mg/kg body weight (20 mL/kg body weight of 0, 12.5, 35, and 100 mg/mL in water for injection, respectively) by gavage. The animals were observed frequently on clinical signs such as abnormalities in external appearance, nutritional condition, posture, behavior, and excrement for the first 6 h after administration (preadministration, immediately, and 5, 15, 30 min, 1, 2, 4, and 6 h after administration), and thereafter once a day for 14 days (day 0: the day of administration). Body weight was measured in the morning on the day of administration and days 1, 2, 3, 7, 10, and 14 after administration. All animals were sacrificed by exsanguination (via the abdominal aorta) under isoflurane anesthesia after the 14 day observation period. External appearance and organs/tissues in the cranial, thoracic, and abdominal cavities were observed macroscopically.

4.9. Lethal Dose and Statistical Analyses. The approximate lethal dose (the minimum lethal dose) was estimated on the basis of the cumulative mortality for 14 days after administration. Body weight was analyzed by multiple comparison. First, analysis of variance was conducted by the Bartlett test (level of significance: 1%). If variances were homogeneous, data were analyzed by the Dunnett test, whereas heterogeneous data were analyzed by the Steel test between the control group and each dose group (levels of significance: 5 and 1%, two-tailed).^{34–36} Analysis was done using SAS Release 9.1.3 (SAS Institute Inc.).

4.10. Ninety Day Oral Gavage Toxicity Study. The oral administration period to the test animals (6 weeks old; $n = 10/\text{sex/group}$) was set at 13 weeks (91 days). The frequency of administration was once a day (7 times per week). The dose volume was set at 10 mL/kg body weight, and test formulations were administered by gavage using flexible stomach tubes. For animals in the control group, the vehicle (water for injection) was administered in the same manner. The dose was set at 0, 500, or 1000 mg/kg (0, 50, or 100 mg/mL; dose volume: 10 mL/kg), and a total of three groups including a control group were provided.

All animals were observed for clinical signs including external appearance, nutritional condition, posture, behavior, and excrement three times a day before dosing, immediately after, and 1–3 h after dosing during the administration period: animals were observed twice a day, before dosing and immediately after dosing, on the days for detailed clinical observation.

The detailed clinical observation, manipulative test, and measurements of grip strength were conducted on all animals in week 12. All animals were weighed three times on days 1, 4, and 7 in week 1 and twice a week thereafter every 3 or 4 days. Food consumption was measured on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91. The ophthalmology examination with an indirect ophthalmoscope (Omega 200, HEINE Optotechnik GmbH & Co, Germany) was done for all animals on day 0 and for 6 animals (with animal numbers 01–06 in each group) in each group on day 90.

On week 13 (days 85–86), after dosing on the day of examination, all animals were individually placed in a wire mesh cage with a urine collector, and 4 h urine was collected under deprivation of feed but with free access to water, and then 20 h urine was collected with free access to feed and water. The urinalysis was carried out with Multistix (Urinary Automatic Analyzer CLINITEK 500, Siemens Healthcare Diagnostics Inc, Germany). Urinary sediments on the 4 h urine samples were examined with a microscope. Osmotic pressure was measured on the 20 h urine samples. One day excretions of sodium, potassium, and chloride were calculated from the 24 h urine volume, and the concentrations were measured.

At the scheduled necropsy on the day following the end of the administration period, all animals were subjected to laparotomy under isoflurane anesthesia after fasting overnight (for approximately 16–21 h) from the previous day, and blood was collected and used for measurement of hematology, prothrombin time, activated partial thromboplastin time, fibrinogen, and blood chemistry.

After collecting blood samples, all the organs and tissues of all animals were carefully necropsied. Then, the organs and tissues of all animals were weighed, and organ weight per 100 g body weight (relative weight) was calculated based on the body weight at the time of necropsy and absolute organ weight. All of the organs and tissues of all animals were fixed and preserved in phosphate buffer containing 10% formalin (v/v), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

4.11. Statistical Analyses. Quantitative data of open field observation, quantitative data of manipulative tests, measurements of grip strength and motor activity, body weight, food consumption, water intake, quantitative data of urinalyses, hematology, blood chemistry, and organ weights were analyzed by multiple comparison. First, analysis of variance was conducted by the Bartlett test (level of significance:

1%).^{34–36} If variances were homogeneous, data were analyzed by the foregoing method.

4.12. Clinical Study of Bangle Extract: Study Design.

The present study was performed by open trial from July to September 2015. The protocol of this study was approved by the institutional ethics committee and conducted according to the guidelines in the Declaration of Helsinki. All procedures involving subjects were approved by the Science Research Center Alternative Medicine Nonprofit Organization.

4.13. Subjects. Sixteen healthy adults, who were judged suitable by the doctor and handed in written consent, were selected as subjects and divided into two groups in this study. The usual ingestion group was five females (21–24 years) and three males (21–23 years). The quintuple group was six males (32–67 years) and two females (43, 50 years).

4.14. Test Schedule. Measurements were taken for blood pressure, pulse rate, blood count, hemogram, biochemical blood tests, and urinalysis from each subject before and after the ingestion. The usual ingestion group ingested one BRE tablet every morning and evening (45.4 mg of BRE and 10 mg of *t*- and *c*-bangles per day) for 4 weeks. The quintuple group ingested five BRE tablets every morning and evening (227 mg of BRE and 50 mg of *t*- and *c*-bangles per day) for 4 weeks. All subjects refrained from ingesting a new health food during the test period. To measure plasma concentrations, blood from each of the five subjects (32–54 years; four males and a female) in the quintuple group was collected in tubes containing EDTA-2K (VENOJec T II glass tube, TERUMO, Japan) at 1, 2, 4, and 24 h after the ingestion of ten BRE tablets on the 28th day, centrifuged to obtain plasma, and maintained at 80 °C.

4.15. Safety Evaluation. Safety was evaluated according to National Cancer Institute CTAE v4.0-Japanese JOCG. Subjective symptoms, blood pressure, pulse rate, blood count, hemogram, biochemical test of blood, and urinalysis were evaluated. Adverse events were defined as all undesirable events including subjective symptoms, abnormal objective findings, and abnormal changes in this test and vital signs.

4.16. Statistical Analyses. Statistical analysis was performed using paired *t*-tests, and differences with *p*-value <0.05 were considered as statistically significant.

4.17. Sample Preparation of Plasma for UPLC-MS/MS Analysis. To 0.2 mL of the subject plasma, 0.2 mL of 2-propanol was added, mixed, and centrifuged for 2 min. The supernatant was passed through an Oasis PRiMe HLB Extraction Cartridge after addition of 10 μL of ultrapure water. This cartridge was washed two times with 0.5 mL of 5% methanol, and then swept two times with 0.5 mL of acetonitrile each time. After evaporation of the acetonitrile eluate with a Conbinievapo, the residue was dissolved in 0.2 mL of acetonitrile and centrifugally filtered through a 0.2 μm filter. Next, 1 μL of the filtrate was injected into the UPLC-MS/MS.

4.18. UPLC-MS/MS Analysis. The obtained filtrate was analyzed by a UPLC system. The *cis*- and *trans*-bangles of the filtrate were separated from the other peaks by using the C18 column at 35 °C. Injection was achieved with an autosampler kept at 10 °C. The mobile phase consisting of acetonitrile and an ultrapure aqueous solution of 0.01% ammonium acetate was used. The gradient system was as follows: 70–98% acetonitrile (0–6 min) and 98% acetonitrile (5 min) at a flow rate of 0.2 mL/min. To re-equilibrate, 70% acetonitrile was flowed through the system for 5 min.

MS/MS was carried out using a Quadrupole Time of Flight mass spectrometer (Waters) with an Electrospray Ionization (ESI) interface. The ESI source was set at the positive ionization mode. The daughter ion ($C_{16}H_{19}O_2$)⁺ m/z 243, which liberated from (M + H)⁺ m/z 381, for the dimethoxyphenylbutadiene dimer, banglene ($C_{24}H_{28}O_4$), was selected as the detecting ion under the collision energy of 15 eV. Data analysis was performed by MassLynx software (Waters, Tokyo).

4.19. Standard and Quality Control Samples. Stock solutions were prepared as standard solutions prepared by diluting the original methanol solution that contained concentrations of *c*- and *t*-banglenes of 0.5 mg/mL each to concentrations of 1, 5, 10, 20, and 50 ng/mL with the addition of methanol. These stock solutions were kept at −40 °C. Sample solutions containing concentrations of 1, 5, 10, 25, and 50 ng/mL each were prepared by adding the original solution to human plasma. Recovery of these sample solutions was 80–120%. Accordingly, the assay was carried out by the absolute calibration method.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02485.

Validation of the determination method by UPLC-MS/MS analysis (Table 1S) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: fukuyama@ph.bunri-u.ac.jp. Tel: +81-88-602-8435. Fax: +81-88-655-3051.

ORCID

Yoshiyasu Fukuyama: 0000-0003-0989-172X

Notes

The authors declare no competing financial interest.

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